Engineering Nitrated Foreign Antigens with Enhanced In Vivo Immunogenicity via Site-Specific Nonstandard Amino Acid Incorporation

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Bacterial pathogens remain an important cause of infectious disease, while effective vaccines are unavailable for many clinically relevant species. Recombinant protein subunit vaccines provide a means of targeting conserved bacterial antigens but can exhibit limited immunogenicity and often require formulation with external adjuvants to generate robust immune responses. Genetic code expansion provides an opportunity to engineer antigen chemistry through site-specific incorporation of nonstandard amino acids. In this work, we investigated incorporation of *para*-nitro-L-phenylalanine (pN-Phe), a nitroaromatic nonstandard amino acid previously shown to alter immune recognition of self-proteins, as a strategy to modulate the immunogenicity of foreign protein antigens.

We first selected *Shigella* invasion plasmid antigen D (IpaD), a conserved antigen with a direct role in pathogenesis, as a model bacterial antigen and developed a preliminary workflow for site selection, recombinant expression, purification, endotoxin control, and characterization of a nitrated IpaD variant Y104pNPhe. We performed a series of pulmonary prime-boost immunization studies in C57BL/6 mice and demonstrated that nitrated IpaD could enhance humoral immune responses relative to wild-type antigen, with increased serum IgG responses observed across independent endotoxin-controlled studies. Increases in mucosal IgG and IgA and serum IgG subclasses were also observed, although these responses exhibited greater within- and between-study variability. An acute 18 h pulmonary immunogenicity study further demonstrated increased neutrophil presence, altered dendritic cell costimulatory marker expression, and increased local TNF-α and B-cell activating factor following nitrated IpaD immunization. In vitro mTLR2 reporter assays additionally demonstrated greater TLR2-associated activity in nitrated IpaD preparations. These data support pN-Phe incorporation at Y104 as a modest immunostimulatory modification while motivating further investigation of its underlying innate and adaptive mechanisms.

Finally, we extended this strategy to influenza hemagglutinin (HA) to evaluate the production and immunogenicity of site-specifically nitrated viral glycoproteins. Mammalian genetic code expansion was established in HEK-derived cells and used to produce multiple nitrated HA variants, with recombinant expression strongly dependent on the site of pN-Phe incorporation. Three variants were advanced to a proof-of-concept mouse immunization study. Although the nitrated HA variants remained immunogenic, nitration at the selected sites did not significantly increase total and stem region-directed serum IgG or hemagglutination-inhibition activity relative to wild-type HA.

Collectively, this dissertation demonstrates that site-specific pN-Phe incorporation can modulate the immunogenicity of a foreign protein antigen but that its effects are context dependent rather than universally applicable. This work establishes a framework for engineering and producing nitrated foreign antigens and provides a foundation for future studies defining mechanism, controlling antigen delivery, and determining whether nitration-associated immunoenhancement can translate into improved vaccine-induced protection.